DATA EVALUATION REPORT

T 99-19

Study Type: Reverse Mutation Assay
OPPTS 870.5100

Prepared for

Antimicrobial Division
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U.S. Environmental Protection Agency
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Contract Number:68-W-01-036
Work Assignment No.: 0248.4000.001.02 TAF 1-2-3
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EPA Reviewer: _____ **Signature:** _____
Registration Branch, Antimicrobials Division **Date** 4/20/05
EPA Secondary Reviewer: _____ **Signature:** _____
Registration Branch, Antimicrobials Division **Date** _____

TXR#:

DATA EVALUATION RECORD

STUDY TYPE: *In vitro* Bacterial Gene Mutation *(Bacterial system, Salmonella typhimurium; E. coli)*/ mammalian activation gene mutation assay; OPPTS 870.5100 [§84-2]; OECD 471.

PC CODE: Not provided

DP BARCODE: 305775
SUBMISSION NO.: Not provided

TEST MATERIAL (PURITY): T 99-19 (40%)

SYNONYMS: Sanitized® T 99-19

CITATION: Thompson, P.W. (2000) Reverse Mutation Assay "Ames Test" Using Salmonella Typhimurium and Escherichia Coli With T 99-19 (40%) Safepharm Laboratories Limited, Derby, United Kingdom. Laboratory report number 502/021. July 13, 2000. MRID 46280412. Unpublished.

SPONSOR: Sanitized AG, Lyssachstraße 95, CH-3401 Burgdorf, Switzerland

EXECUTIVE SUMMARY: In a reverse gene mutation assay in bacteria (MRID 460280412), strains TA98, TA100, TA1535, and TA1537 of *S. typhimurium*, and *E. coli* WP2*uvr*A$^-$ were exposed to T 99-19 (40% pure, Batch 232) in the presence and absence of mammalian metabolic activation, S9, from Phenobarbitol/β-Naphthoflavone -induced male Sprague-Dawley rats. In each of two main assays, the TA98, TA100, TA1535, and TA1537 strains were exposed to T 99-19 at concentrations of 5, 15, 50, 150, 500, and 1,500 μg/plate in the absence of S9 and at concentrations of 15, 50, 150, 500, 1,500, and 5,000 μg/plate in the presence of S9. The WP2*uvr*A$^-$ strain was exposed to T 99-19 at concentrations of 15, 50, 150, 500, 1,500, and 5,000 μg/plate in the presence and absence of S9. All exposures were conducted using the plate incorporation method. Triplicate plates per dose were tested with concurrent positive and negative controls. A range-finding assay in TA100 and WP2*uvr*A$^-$ (0.15-5,000 μg/plate) was used to determine the doses for the main mutation assay.

In the preliminary range-finding assay, T 99-19 was tested to determine appropriate dose levels for the main assays. Complete toxicity was first observed at 1,500 μg/plate in the TA100, -S9 experiment and at 5,000 μg/plate in the other experiments. The results of the main assay found no increase in the number of mean revertants over the negative control at any condition. Partial or complete absence of background lawns was noted at the highest doses (1,500 or 5,000 μg/plate) in all strains, ±S9. Toxicity began in strains TA100 and TA 1535 at 500 μg/plate, ±S9 (both main experiments). In strain WP2*uvr*A$^-$, toxicity began at 5,000 μg/plate in the presence of S9 in both main experiments and in the absence of S9 in the first experiment. In the second experiment in the absence of S9, toxicity began at 1,500 μg/plate. For

strain TA98, toxicity began at 1,500 μg/plate in the absence of S9 in both main experiments and in the presence of S9 in the second experiment. In the first experiment in the presence of S9, toxicity began at 5,000 μg/plate. For strain TA1537, toxicity began at 1,500 μg/plate, ±S9 (both main experiments). The positive controls induced the appropriate responses in the corresponding strains. There was no evidence of induced mutant colonies over background in any strain, ±S9, at any concentration tested.

This study is classified as **ACCEPTABLE (GUIDELINE)** and satisfies the guideline requirement for the requirement for Test Guideline OPPTS 870.5100; OECD 471 for *in vitro* mutagenicity (bacterial reverse gene mutation) data.

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: Sanitized T 99-19

Description:	Clear, yellow liquid (received on May 26, 2000) stored in the dark at room temperature.
Lot/Batch #:	Batch No. 232
Purity:	40% a.i.
CAS # of TGAI:	Not provided
Structure:	Not provided
Solvent Used	None

2. Control Materials:

Negative:	Sterile distilled water (vehicle control)
Solvent (final conc'n):	N/A
Positive:	Nonactivation:
	N-ethyl-N-nitro-N-nitrosoguanidine (ENNG): 3 μg/plate for TA100, 5 μg/plate for TA1535 and 2 μg/plate for WP2*uvr*A$^-$
	9-Aminoacridine (9AA): 80 μg/plate for TA1537
	4-Nitroquinoline-1-oxide (4NQO): 0.2 μg/plate for TA98
	Activation:
	2-Aminoanthracene (2AA): 1 μg/plate for TA100, 2 μg/plate for TA1535 and TA1537 and 10 μg/plate for WP2*uvr*A$^-$
	Benzo(a)pyrene (BP): 5 μg/plate for TA98

3. Activation: S9 derived from:

X	induced		Aroclor 1254	X	Rat	X	Liver

	non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other: β-Naphthoflavone		Other		

The rat liver S9 homogenate was prepared from male Sprague-Dawley rats (~250 g) that each received three consecutive daily doses of Phenobarbitol/β-Naphthoflavone (80/100 mg/kg/day) prior to S9 preparation. Before use, each batch of S9 was assayed for its ability to metabolize the indirect mutagens 2 -Aminoanthracene and Benso(a)pyrene. The S9 was stored at -196°C.

The S9 mix was prepared immediately before using sterilized co-factors and maintained on ice for the duration of the test. The S9 cofactor solution consisted of the following components: 5.0 mL of S9, 1.0 mL of 1.65 M KCl/0.4 M $MgCl_2$, 2.5 mL of 0.1 M Glucose-6-phosphate, 2.0 mL of 0.1 M NADPH, 2.0 mL of NADH, 25.0 mL of 0.2 M sodium phosphate buffer (pH 7.4), and 12.5 mL of sterile distilled water.

To assess the sterility of the S9-mix, a 0.5 mL aliquot of the S9-mix and 2 mL of molten, trace histidine or tryptophan supplemented, top agar was overlaid onto a sterile agar plate. This procedure was repeated in triplicate on the day of each experiment.

4. Test organisms: *S. typhimurium* strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538	X	*E.coli* WP2uvrA$^-$		

Properly maintained?	X	Yes		No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	X	Yes		No

5. Test compound concentrations used:
The test compound concentrations evaluated are presented in Table 1.

Test	Bacterial Strains Evaluated	With or Without Metabolic Activation	Test Substance Concentrations Evaluated	No. of Plates/ Dose
Preliminary Cytotoxicity Test (Plate Incorporation)	TA100 and WP2*uvr*A$^-$	-S9 and +S9	0, 0.15, 0.5, 1.5, 5, 15, 50, 150, 500, 1,500, and 5,000 μg/plate	1

Table 1. Evaluated Test Compound Concentrations				
Test	Bacteria Strains Evaluated	With or Without Metabolic Activation	Test Material Concentration (Corresponding Active Ingredient Concentration)	No. of Plates per Dose
Main Test I and II (Plate Incorporation)	TA98, TA100, TA1535, TA1537	-S9	5, 15, 50, 150, 500, and 1,500 μg/plate	3
		+S9	15, 50, 150, 500, 1,500, and 5000 μg/plate	
	WP2*uvr*A⁻	-S9 and +S9	15, 50, 150, 500, 1,500, and 5000 μg/plate	

B. TEST PERFORMANCE

1. Type of *Salmonella* assay:

X standard plate test
_ pre-incubation (20 minutes)
_ "Prival" modification *(i.e. azo-reduction method)*
_ spot test
_ other

2. Protocol:

Tests were performed using the direct plate incorporation method. Top agar was prepared using 0.6% Difco Bacto agar (lot number 1423111 01/02) and 0.5% sodium chloride with 5 mL of 1.0 mM histidine and 1.0 mM biotin or 1.0 mM tryptophan solution added to each 100 mL of top agar. Vogel-Bonner Minimal agar plates were prepared using 1.6% Oxoid Agar Technical No. 3 (lot number 810616-2 1/05) with Vogel-Bonner Medium E and 20 mg/mL D-glucose.

Measured aliquots (0.1 mL) of one of the bacterial cultures were dispensed into sets of test tubes followed by 2.0 mL of molten top agar (trace histidine or tryptophan supplemented); 0.1 mL of the test material formulations, vehicle control or positive control; and 0.5 mL of S9-mix or phosphate buffer. The contents of each test tube were mixed and equally distributed onto the surface of Vogel-Bonner Minimal agar plates (one tube per plate). This procedure was repeated, in triplicate, for each bacterial strain and for each concentration of test material with and without S9-mix.

All of the plates were incubated at 37°C for approximately 48 hours and the frequency of relevant colonies assessed using a Domino colony counter.

3. Statistical Analysis: Dunnett's method of linear regression was used to determine if there was a

significant increase in the relevant count in at least one strain of bacteria.

4. Evaluation Criteria:

A test was considered acceptable if: (1) All tester cultures exhibit a characteristic number of spontaneous reverants per plate in the vehicle and untreated controls. Acceptable ranges and historical control data (1998 and 1999) are presented in the Study Report; (2) The appropriate characteristics for each tester strain have been confirmed (e.g. rfa cell-wall mutation and pkM101 plasmid R-factor, etc.); (3) All tester strain cultures should be in the approximate range of 1 to 9.9 a 10^9 bacteria per mL; (4) Each mean positive control values should be at least two times the respective vehicle control value for each strain, thus demonstrating both the intrinsic sensitivity of the tester strains to mutagenic exposure and the integrity of the S9-mix. The historical positive ranges for 1998 and 1999 are presented in the Study Report; (5) There should be a minimum of four non-toxic test material dose levels; and (6) There should be no evidence of excessive contamination.

A response was considered positive if: (1) the test material induced a reproducible, dose-related and statistically (Dunnett's method of linear regression) significant increase in the reverant count in at least one strain of bacteria.

II. REPORTED RESULTS

A. PRELIMINARY CYTOTOXICITY ASSAY: The preliminary assay evaluated the test article at a dose range of 0.15-5000 μg/plate, ±S9, using TA100 and WP2uvrA- strains. For the experiments using TA100, toxicity (partial or complete absense of bacterial background lawn) was first observed at 500 μg/plate for plates without S9 and 1,500 μg/plate for plates with S9. For the experiments conducted using WP2uvrA-, toxicity (partial or complete absence of bacterial background lawn) was first observed at 1,500 μg/plate for plates without S9 and 5,000 μg/plate for plates with S9.

B. MUTAGENICITY ASSAY: The results of the main mutagenicity assays are presented below in Table 2. The main assays tested 6 dose levels of the test article in triplicate. Triplicate plates of the positive and negative control were tested concurrently. The requirements for an acceptable assay were met. No significant increase in the number of mean colonies per plate was observed, ±S9. No precipitate was observed in any strain at any condition. The solvent controls were within the appropriate historical ranges, and the positive controls were significantly increased over the solvent controls, which verified the sensitivity of the assay.

According to the text in the Study Report, the test material caused a visible reduction in the growth of the bacterial lawn to all of the test strains, initally at 500 μg/plate without S9 and 1,500 μg/plate with S9. Based on more detailed data reported in Tables 2 through 5 of the Study Report, partial or complete absence of bacterial background lawn in the experiments without S9 was first observed at 500 μg/plate for TA100 and TA1535 (both experiments). For the TA98 and TA1537 strains (-S9), partial or complete absence of the bacterial background lawn was first observed at 1,500 μg/plate in both experiments. For WP2uvrA- (-S9), the partial or complete absence of bacterial background lawn was first observed at 5,000 μg/plate in the first experiment and 1,500 μg/plate in the second experiment. For the experiments conducted in the presence of S9, the partial or complete absence of bacterial background lawn was first observed in both experiments at 1,500 μg/plate for the TA100, TA1535, and TA1537 strains. For the

TA98 strain, the partial or complete absence of the bacterial lawn was first observed at 5,000 μg/plate in the first experiment and 1,500 μg/plate in the second experiment. For WP2uvrA-, the partial or complete absence of the bacterial lawn was first observed at 5,000 μg/plate in both experiments. The positive controls exhibited a clear, positive response, ±S9.

Table 2. Revertant Counts of Main Assays

Substance	Dose Level (μg/plate)	TA100		TA1535		WP2uvrA		TA98		TA1537	
		Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
First Experiment, -S9											
Negative Control	0	103	7.6	24	4.0	21	2.6	16	3.1	7	1.2
Test Article	5	88	11.2	19	3.0	NT	NT	14	2.3	7	1.0
	15	97	2.0	19	1.5	17	7.6	21	7.5	11	5.1
	50	110	9.6	20	2.5	19	5.3	19	4.2	8	4.0
	150	83	2.5	21	4.2	19	6.4	15	1.0	8	0.6
	500	**64**	**13.8**	**14**	**4.0**	19	1.2	22	4.2	11	1.2
	1500	**0**	**0.0**	**6**	**1.5**	19	3.5	**14**	**3.1**	**10**	**2.1**
	5000	NT	NT	NT	NT	**0**	**0.0**	NT	NT	NT	NT
Positive Control	3 (ENNG)	343	25.4	--	--	--	--	--	--	--	--
	5 (ENNG)	--	--	172	15.2	--	--	--	--	--	--
	2 (ENNG)	--	--	--	--	251	23.5	--	--	--	--
	2 (4NQO)	--	--	--	--	--	--	162	12.2	--	--
	80 (9AA)	--	--	--	--	--	--	--	--	475	35.2
First Experiment, +S9											
Negative Control	0	88	4.6	13	3.1	19	7.2	30	6.0	12	1.5
Test Article	15	88	11.8	11	0.6	21	8.7	35	3.2	13	4.0
	50	88	4.0	14	2.1	25	0.6	23	4.6	14	1.5
	150	73	7.0	15	3.8	20	2.9	35	4.0	12	2.6
	500	61	4.0	15	2.6	20	5.5	28	0.6	11	2.3
	1500	**26**	**2.1**	**8**	**1.5**	19	2.5	27	6.7	**10**	**2.9**
	5000	**0**	0.0	**0**	0.0	**7**	**3.1**	**0**	**0.0**	**0**	**0.0**
Positive Control	1 (2AA)	1866	175.8	--	--	--	--	--	--	--	--
	2 (2AA)	--	--	199	77.1	--	--	--	--	213	78.6
	10 (2AA)	--	--	--	--	347	16.5	--	--	--	--
	5 (BP)	--	--	--	--	--	--	409	30.9	--	--
Second Experiment, -S9											
Negative Control	0	109	12.1	30	1.2	26	4.9	23	6.1	10	5.1

Table 2. Revertant Counts of Main Assays											
Substance	Dose Level (μg/plate)	TA100		TA1535		WP2uvrA		TA98		TA1537	
		Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
Test Article	5	99	4.5	25	1.2	NT	NT	16	8.4	9	2.0
	15	106	4.9	31	3.8	25	8.0	21	4.7	11	2.5
	50	108	5.0	27	2.9	27	3.5	25	2.9	10	1.5
	150	104	3.5	27	2.9	30	4.9	20	1.7	13	3.6
	500	**83**	**9.0**	**21**	**6.4**	30	3.0	21	4.6	11	4.0
	1500	**0**	**0.0**	**4**	**2.0**	**18**	**6.1**	**13**	**1.5**	**8**	**2.3**
	5000	NT	NT	NT	NT	**0**	**0.0**	NT	NT	NT	NT
Positive Control	3 (ENNG)	488	56.4	--	--	--	--	--	--	--	--
	5 (ENNG)	--	--	546	30.5	--	--	--	--	--	--
	2 (ENNG)	--	--	--	--	725	63.7	--	--	--	--
	2 (4NQO)	--	--	--	--	--	--	177	13.8	--	--
	80 (9AA)	--	--	--	--	--	--	--	--	942	132.5
Second Experiment, +S9											
Negative Control	0	129	4.4	20	4.0	25	3.5	38	6.0	14	1.5
Test Article	15	115	9.5	21	4.0	31	2.0	34	3.5	13	4.2
	50	129	9.1	23	2.3	25	3.0	42	2.1	14	1.5
	150	109	14.1	23	5.5	27	4.7	32	1.5	18	2.6
	500	86	14.2	16	5.0	29	9.0	39	4.6	17	0.0
	1500	**31**	**3.6**	**10**	**3.1**	26	5.6	34	0.0	16	1.7
	5000	**0**	**0.0**	**0**	**0.0**	**5**	**5.0**	0	0.0	0	0.0
Positive Control	1 (2AA)	1867	252.8	--	--	--	--	--	--	--	--
	2 (2AA)	--	--	391	24.9	--	--	--	--	533	42.3
	10 (2AA)	--	--	--	--	788	73.9	--	--	--	--
	5 (BP)	--	--	--	--	--	--	400	23.0	--	--

Calculated by Versar using data from Tables 2-5, report pgs. 17-20.
NT = Not tested
Bold = partial or complete absence of bacterial background lawn, according to study author.

III. DISCUSSION and CONCLUSIONS

A. INVESTIGATORS' CONCLUSIONS: The test article was determined not to be mutagenic at any dose or in any strain, ±S9.

B. REVIEWER COMMENTS: The study results clearly indicate that the test article was not mutagenic, but that it induced toxicity at the top one or two dose levels evaluated. Appropriate methods, protocols, strains of bacteria, acceptance criteria, and criteria for a positive response were used.

C. STUDY DEFICIENCIES: Only minor deficiencies in the study are noted, which do not affect the guideline acceptability of the study. These include the following:

- lack of verification of test article concentrations;
- lack of reporting of stability and solubility of the test article;
- use of 2-aminoanthracene as the sole positive control compound for some strains, +S9;
- lack of reporting of bacterial cell density prior to plating.

D. STUDY CLASSIFICATION: This study is classified **ACCEPTABLE-GUIDELINE** and meets the guideline requirements for an *in vitro* bacterial gene mutation *(Salmonella typhimurium* and *E. coli)*/mammalian activation gene mutation assay; OPPTS 870.5100 [§84-2].